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Receptor-selective determinants in catfish gonadotropin seat-belt loops

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Abstract

Mammalian gonadotropins are highly selective. Charge differences between the Cys^{10-11} sequence of FSHβ and LHβ/CGβ seat-belt loops determine the ability of these hormones to interact with the LH-R. Selective FSH-R binding is mainly dependent on the presence of an FSHβ-specific sequence between Cys^{11-12} of the seat-belt loop. Intriguingly, African catfish LHβ (cfLHβ) lacks a positively charged Cys^{10-11} region and stimulates both catfish LH-R and FSH-R with comparable potencies. Our studies on the promiscuous behaviour of cfLH using chimeric gonadotropins revealed that the Cys^{10-11} region of cfLHβ contains cfLH-R-selective determinants, whereas the Cys^{11-12} region of cfLHβ confers FSH-R-stimulating activity to cfLH. Hence, the location of receptor-selective determinants appeared to be fairly well conserved throughout evolution, despite the low sequence identity between mammalian and catfish seat-belt loops. Moreover, various structure–function differences between gonadotropins are discussed in the context of the different (female) reproductive strategies between mammalian and non-mammalian species that required the divergence to a more specific LH-R-stimulating activity of one of the gonadotropins in mammals.

Keywords: Luteinizing hormone; Follicle-stimulating hormone; Hormone selectivity

1. Introduction

In mammals, gonadal function is regulated by two distinct, but complementary acting, pituitary-derived gonadotropins (follicle-stimulating hormone, FSH, and luteinizing hormone, LH) via the specific activation of their respective receptors (FSH-R and LH-R). LH and FSH, together with thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG), form the family of glycoprotein hormones, which are glycosylated heterodimeric molecules, each composed of a non-covalent association of a common α-subunit with a hormone specific β-subunit. Apart from LH and CG that both are able to activate the LH-R in primates and equids, the normal interaction between the glycoprotein hormones and their respective receptors are highly specific (<0.1% cross-reactivity; Campbell et al., 1997; Vischer et al., 2003a). In contrast to mammalian gonadotropins, their counterparts in fish display reduced receptor selectivity. For example, purified, pituitary-derived catfish LH (cfLH) as well as recombinant cfLH (rcfLH) can activate both the catfish LH receptor (cfLH-R; Vischer and Bogerd, 2003a) and catfish FSH receptor (cfFSH-R; Bogerd et al., 2001) with comparable potencies. However, cfLH is not able to activate the catfish TSH-R (Vischer and Bogerd, 2003b). Moreover, recombinant catfish FSH (rcfFSH) predominantly activates the cfFSH-R (Vischer et al., 2003b).

Crystallographic analyses of deglycosylated hCG and hFSH (Lapthorn et al., 1994; Fox et al., 2001) suggested a similar overall folding of the common α-subunit, but also of the two gonadotropin β-subunits, each consisting of a cystine knot architecture that divides each subunit into three elongated antiparallel loops (i.e. α1, α2, α3; β1, β2, β3). Although the primary β-subunit sequences have diverged suffi-

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ciently during evolution to confer specificity to each of these heterodimeric glycoprotein hormones (Li and Ford, 1998), their β-subunits are structurally very similar due to the positional conservation of 12 conserved cysteine residues. Six cysteines form the cystine knot motif, while the remaining six residues form three additional loop-stabilizing disulfide bridges.

Based on sequence differences between distinct β-subunits, chimeric analogs have been generated in order to identify β-subunit regions that confer receptor-specificity to these hormones. These studies revealed that the region between the 10th and 12th conserved cysteine residues (Cys^{10-12}; i.e. the seat-belt region) of each glycoprotein hormone β-subunit is critically involved in determining specificity for its respective receptor. In particular, the net charge differences in the determinant loop (i.e. the region between Cys^{10} and Cys^{11}) of the seat-belt region between mammalian LH/CG β-subunits on the one hand, and FSH/TSH β-subunits on the other hand are thought to have partially separated LH-R- from FSH-R/TSH-R-activating properties (Han et al., 1996; Campbell et al., 1997). It is thought that additional sequence divergence in the carboxy-terminal seat-belt segment (i.e. between Cys^{11} and Cys^{12}) as well as outside the seat-belt region has further separated specific lutropic, follitropic and thyrotropic activities of these hormones to their respective natural receptors (Grossmann et al., 1997).

In contrast to the situation in mammalian gonadotropins, inspection of the determinant loop of fish gonadotropins revealed the absence of striking net charge differences between their LH and FSH β-subunits. To elucidate if and how the seat-belt region of cfLHβ contributes to the observed promiscuous receptor activation (Bogerd et al., 2001; Vischer and Bogerd, 2003a), we studied the effects of several substitutions of the intercysteine segments of the cfLHβ seat-belt region in order to identify sequence information necessary to specifically stimulate the cfFSH-R and the cfLH-R.

2. Material and methods

2.1. Construction and expression of cassette-substituted gonadotropin cDNAs

Chimeric seat-belt cfLH β-subunit (cfLHβ) constructs were generated by cassette-substitution of individual intercysteine segments of the seat-belt region or the entire seat-belt region between the 10th and 12th conserved cysteine residues (i.e. Cys^{90} and Cys^{107}, respectively) with the corresponding hCG-regions or cfFSH-regions, or with the corresponding number of Ala residues (Fig. 1). In addition, reciprocal chimeras were made by introducing the cfLH seat-belt region sequences into the cfFSH β-subunit (cfFSHβ) (Fig. 1). To this end, the cDNAs encoding the cfFSHβ (GenBank accession no. AF324541) or the cfLHβ (GenBank accession no. X97761), in which the first 30 bases of their leader sequences were optimized for *Dictyostelium discoideum* codon

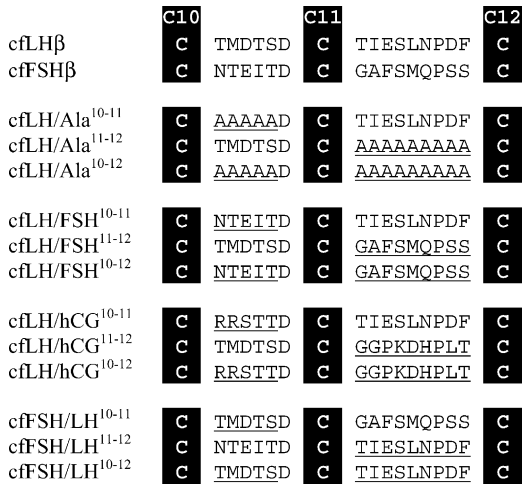

Fig. 1. Wild type and mutant cfLH and cfFSH β-subunit seat-belt amino acid sequences. The amino acid sequences of the seat-belt region between the conserved Cys^{10} and Cys^{12} of cfLHβ and cfFSHβ are shown. Substitutions of the determinant loop (between Cys^{10} and Cys^{11}) and/or C-terminal segment (between Cys^{11} and Cys^{12}) of the seat-belt region of cfLHβ with Ala-cassettes (cfLH/Ala), corresponding cfFSHβ (cfLH/FSH) or hCGβ (cfLH/hCG) segments are shown, of which the mutated segments are underlined. In addition, cfFSHβ/cfLHβ (cfFSH/LH) chimeras are shown.

usage, and preceded by a *Dictyostelium* consensus translation initiation sequence (AAAAA; Vervoort et al., 2000) and flanked by *Bgl*II and *Spe*I restriction endonuclease sites at their 5′- and 3′-ends, respectively (Vischer et al., 2003b), were used as templates for PCR-based mutagenesis (Ali and Steinkasserer, 1995). Briefly, two cDNA fragments were generated using sense and antisense mutation primers in combination with specific primers demarcating the 3′- and 5′-end of the open-reading frame, respectively, on the above mentioned templates. Next, these two PCR products were used in a self-primed fusion PCR reaction. The fusion products were subsequently PCR amplified using specific primers demarcating the open-reading frame. All PCR products generated were TOPO-cloned into pcDNA3.1/V5-His plasmid (Invitrogen) for sequence analysis. The mutant seat-belt β-subunit cDNA inserts were then transferred into the *Dictyostelium* extra-chromosomal expression vector MB12neo, using their *Bgl*II and *Spe*I endonuclease restriction sites (Linskens et al., 1999).

Mutant gonadotropins were recombinantly expressed in the soil amoeba *Dictyostelium discoideum* (strain AX3) and purified as described previously (Vischer et al., 2003b). Briefly, 1 μg of MB12n vector, containing the catfish glycoprotein hormone α-subunit cDNA (GenBank accession no. X97760; cfGPα/MB12n), in combination with 1 μg of mutant β-subunit/MB12neo circular plasmids were cotransformed in 10^7 cells by electroporation. Next, the cells were seeded in 9 cm culture plates. Selection for the α-subunit-containing construct with Blasticidin S (10 μg/ml; ICN, Zoetermeer, The Netherlands) was initiated 5 h after electroporation. After 24 h, the medium was replaced

with fresh medium containing both Blasticidin S (10 mg/ml) and neomycin antibiotic G418 (5 mg/ml; Invitrogen), selecting cells harboring both the cfGPα/MB12n and β-subunit/MB12neo plasmids. Medium was refreshed every 3–4 days, while maintaining selective conditions. Approximately 6 ml of medium was harvested every 3.5 days, in total 3–4 times, starting 10–14 days after electroporation. Harvested medium was stored at −80 °C until used for experimentation.

The recombinant catfish mutant gonadotropins were ∼20-fold concentrated by ultrafiltration, while simultaneously replacing the axenic *Dictyostelium* medium by DMEM-Hepes (Sigma, St. Louis, MI), using Ultrafree-15 Biomax-30 centrifugal devices (Amicon, Millipore Corparation, Bedford, MA). Hormone concentrations were quantified by radioimmunoassays using antisera against intact cfLH and the cfGPα-subunit as described previously (Schulz et al., 1995).

2.2. In vitro gonadotropin receptor activation

In vitro bioactivities of the chimeric catfish gonadotropins were determined on human embryonic kidney T 293 (HEK-T 293) cells expressing the cfFSH-R or the cfLH-R, as described previously (Bogerd et al., 2001; Vischer and Bogerd, 2003a). Briefly, approximately 5×10^6 HEK-T 293 cells were transiently cotransfected with 10 μg of a pCRE/β-gal reporter-gene construct containing a β-galactosidase gene driven by five cAMP responsive elements (Chen et al., 1995) and either 1 μg of the human LH-R (kindly provided by Dr. E. Milgrom, Institut National de la Sante et de la Recherche Medicale, La Kremlin-Bicetre, France), 1 μg of the cfFSH-R or 10 ng of the cfLH-R expression vector constructs. One-hundred-fold less expression vector construct of the constitutively active cfLH-R compared with the cfFSH-R was used for transfection. This reduces the cfLH-R-mediated basal cAMP levels allowing the dose-response curves to fit within the measuring range of the reporter-gene assay (Vischer and Bogerd, 2003a). After 16–18 h, cells were transferred to 96-well plates (∼2.5×10^5 cells/well). The next day, the transfected cells were stimulated for 6 h with various concentrations of each of the mutant catfish gonadotropins in 25 ml DMEM-Hepes containing 0.1% BSA (Sigma). The ligand-induced β-galactosidase activity was measured, and the hormone concentrations inducing half-maximal stimulation (EC_{50}) were calculated using the Graphpad PRISM3 software package (GraphPad Software Inc., San Diego, CA). All experiments were repeated at least 3 times using cells from independent transfections and recombinant hormones derived from different batches.

2.3. Statistics

All results are expressed as the mean ± S.E.M. To evaluate the effect of increasing hormone concentrations on cfLH-R- or cfFSH-R-mediated signaling over basal, the β-galactosidase activity was subjected to analysis of variance (ANOVA) using Statview 4.5 (Abacus Concepts, Berkeley, CA). The ANOVA was followed by a Fisher's probable least-squares difference test to identify significant differences ($P < 0.05$) between individual groups. The same analysis or the student *t*-test ($P < 0.05$) was used to compare the log-transformed half-maximal stimulation (EC_{50}) of the cfFSH-R- or cfLH-R-mediated signal transduction, induced by the recombinant catfish gonadotropins.

3. Results

3.1. Catfish gonadotropin expression in Dictyostelium

Recombinant mutant gonadotropins, expressed in *Dictyostelium discoideum*, were first functionally analyzed (see below), and a posteriori quantified by radioimmunoassay in order to limit the storage time between the concentration procedure and the functional assays. Recombinant gonadotropin levels were measured by two different radioimmunoassays using either a polyclonal antibody against intact cfLH, or a polyclonal antibody against the cfGP α-subunit, as described previously (Vischer et al., 2003b). Typically, the amount of wild type rcfLH was ∼2.2 ± 0.11-fold lower in the cfGPα radioimmunoassay than in the intact cfLH radioimmunoassay, as cfGPα contributes to approximately half of the total molecular weight of intact heterodimeric cfLH (Koide et al., 1992; Rebers et al., 1997). However, all chimeric cfLH analogs, in particular cfLH/Ala^{11-12}, cfLH/Ala^{10-12}, cfLH/hCG^{11-12} and cfLH/hCG^{10-12}, had intact hormone/cfGPα ratios lower than 2.2 (Table 1). Since less than 0.1% of free cfGPα subunit is recovered after ultrafiltration (data not shown), low intact hormone/cfGPα ratios are likely to reflect mutation-induced alterations in the overall heterodimeric analog conformation, rather than a surplus of free cfGPα subunits due to impaired subunit association. In the absence of a specific cfFSH radioimmunoassay, the recombinant cfFSH and cfFSH analog levels were calculated from the anti-cfGPα measurements as described previously (Vischer et al., 2003b), without taking into account possible mutation-induced conformational changes. For convenience, all recombinant wild type and chimeric catfish LH and FSH amounts were quantified using the cfGPα measurements and corrected for their heterodimeric stoichiometry.

3.2. Bioactivities of the chimeric recombinant catfish gonadotropins

The bioactivities of the chimeric hormones were evaluated by measuring their potency to induce specific receptor-mediated signaling in HEK-T 293 cells, transiently transfected with either the cfLH-R or the cfFSH-R. To examine whether promiscuous receptor binding of cfLH is confined to one or both of the two intercysteine modules of its β-subunit seat-belt loop, the individual β-subunit Cys^{10-11} and Cys^{11-12} regions as well as the entire β-subunit seat-belt re-

Table 1
Summary of the intact cfLH/cfGPα ratios and bioactivities of wild type cfLH and cfFSH and mutant β-subunit seat-belt cfLH and cfFSH analogs on HEK-T 293 cells, transiently expressing the cfLH-R or the cfFSH-R

Hormone analogs	Seat-belt sequence		RIA cfLH/cfGPα	cfLH-R cAMP EC_{50} (ng/ml)	cfFSH-R cAMP EC_{50} (ng/ml)
	C^{10}–C^{11}	C^{11}–C^{12}			
rcfLH	cfLH	cfLH	2.2	45.50 ± 14.46[a]	7.61 ± 1.29[b]
cfLH/Ala^{10-11}	Ala	cfLH	1.13	>300*	3.03 ± 1.05[b]
cfLH/Ala^{11-12}	cfLH	Ala	0.36	NS	>100*
cfLH/Ala^{10-12}	Ala	Ala	0.46	NS	>100*
cfLH/hCG^{10-11}	hCG	cfLH	1.72	19.55 ± 4.83[a]	1.59 ± 0.30[c]
cfLH/hCG^{11-12}	cfLH	hCG	0.92	>100*	>300*
cfLH/hCG^{10-12}	hCG	hCG	0.05	>300*	NS
cfLH/FSH^{10-11}	cfFSH	cfLH	1.95	>300*	0.41 ± 0.07[c]
cfLH/FSH^{11-12}	cfLH	cfFSH	1.54	>300*	0.70 ± 0.11[c]
cfLH/FSH^{10-12}	cfFSH	cfFSH	1.75	>300*	0.13 ± 0.13[c]
rcfFSH	cfFSH	cfFSH		>300*	0.29 ± 0.04[c]
cfFSH/LH^{10-11}	cfLH	cfFSH		NS	NS
cfFSH/LH^{11-12}	cfFSH	cfLH		>1000*	1.70 ± 0.28[c]
cfFSH/LH^{10-12}	cfLH	cfLH		>1000*	NS

Recombinant gonadotropin levels were measured using two different RIAs as described under Section 2. The ratio between the amounts of intact cfLH hormone and cfGPα was used as an index to assess hormone conformation, with ratios lower than 2.2 suggesting an mutation-induced impaired hormone conformation. The cAMP production upon stimulation with wild type or mutant cfLH and cfFSH analogs was measured in HEK-T 293 cells transiently cotransfected with either the cfLH-R or the cfFSH-R expression vector construct and a plasmid (pCRE/β-gal) containing a β-galactosidase gene under control of a promoter containing five cAMP-response elements. Due to the limited amounts of analogs produced by *Dictyostelium discoideum* and due to reduced activities of certain analogs, it was not always possible to calculate their half-maximal stimulation (EC_{50}). Hence, analog concentrations that were at least required to induce half-maximal stimulation, were estimated for these cases and are indicated by an asterisk. All data represent an average of at least three independent receptor-stimulation assays with triplicate observations each, and EC_{50} values are given as mean ± S.E.M. [a,b,c]Values sharing the same letter do not differ significantly (see Section 2). NS: no significant stimulation of receptor-mediated cAMP production.

gion (Cys^{10-12}) were replaced with Ala-residues excluding the cysteine residues Cys^{10}, Cys^{11} and Cys^{12}. Since the Asp-residue preceding the 11th Cys residue is highly conserved in all glycoprotein hormones and has been shown to be crucial for their activity (Chen et al., 1991; Grossmann et al., 1997), this residue (i.e. Asp^{96} in cfLHβ) was also excluded in the mutagenesis procedure to create the chimeric gonadotropins (Fig. 1). Replacing Cys^{10-11} of the cfLHβ seat-belt with Ala-residues (cfLH/Ala^{10-11}) significantly reduced its cfLH-R-stimulating activity, but did not affect its cfFSH-R-stimulating activity (Fig. 2A and Table 1). However, Ala-cassettes substitutions between Cys^{11-12} (cfLH/Ala^{11-12}) and the entire seat-belt (cfLH/Ala^{10-12}), substantially reduced the cfLH-R- and cfFSH-R-stimulating activities for both types of chimeric hormones.

To determine more conclusively whether the native seat-belt sequence of cfLHβ is important for its cfFSH-R-stimulating activity, cfLH analogs were generated that harbor seat-belt sequences of hCG. Since hCG had been shown to activate the cfLH-R (Vischer and Bogerd, 2003a), but not the cfFSH-R (Bogerd et al., 2001), the resulting cfLH/hCG chimeras were expected to maintain their cfLH-R-stimulating activity, while being devoid of any cfFSH-R-stimulating activity. Surprisingly, however, substitution of the Cys^{10-11} sequence of cfLHβ with the corresponding sequence of hCGβ (cfLH/hCG^{10-11}) increased the cfFSH-R-stimulating activity of the cfLH chimera to a potency that is similar to rcfFSH, whereas its cfLH-R-stimulating activity was not affected (Fig. 2B and Table 1). Introduction of the hCGβ Cys^{11-12} region into cfLHβ (cfLH/hCG^{11-12}) resulted in at least 40- and 2-fold reduced cfFSH-R- and cfLH-R-stimulating activities, respectively (Fig. 2B and Table 1). Substitution of the entire seat-belt with hCGβ sequences (cfLH/hCG^{10-12}) abolished all measurable cfFSH-R-stimulating activity, while its cfLH-R-stimulating activity was significantly attenuated (Fig. 2B and Table 1). Neither rcfLH nor the cfLH/hCG chimeras were able to induce hLHR-mediated cAMP production over basal (data not shown).

In addition, the seat-belt segments of cfLHβ were substituted with the corresponding sequences of cfFSHβ. Recombinant cfFSH had been shown to have a significantly lower potency for the cfLH-R compared with cfLH (Vischer et al., 2003b). Substitution of the cfLHβ seat belt with the entire cfFSHβ seat-belt sequences (cfLH/FSH^{10-12}), or the Cys^{10-11} and Cys^{11-12} intercysteine segments of cfFSHβ (cfLH/FSH^{10-11} and cfLH/FSH^{11-12}, respectively) resulted in a significant reduction of their cfLH-R-stimulating activities comparable to the intrinsic activity of rcfFSH to stimulate the cfLH-R (Fig. 2C and Table 1). Their abilities to activate the cfFSH-R, on the other hand, were increased to potencies similar to wild type rcfFSH (Fig. 2C and Table 1). In contrast, all reciprocal chimeras, in which cfFSHβ seat-belt segments were replaced by their corresponding cfLHβ sequences (i.e. cfFSH/LH^{10-11}, cfFSH/LH^{11-12} and cfFSH/LH^{10-12}), ac-

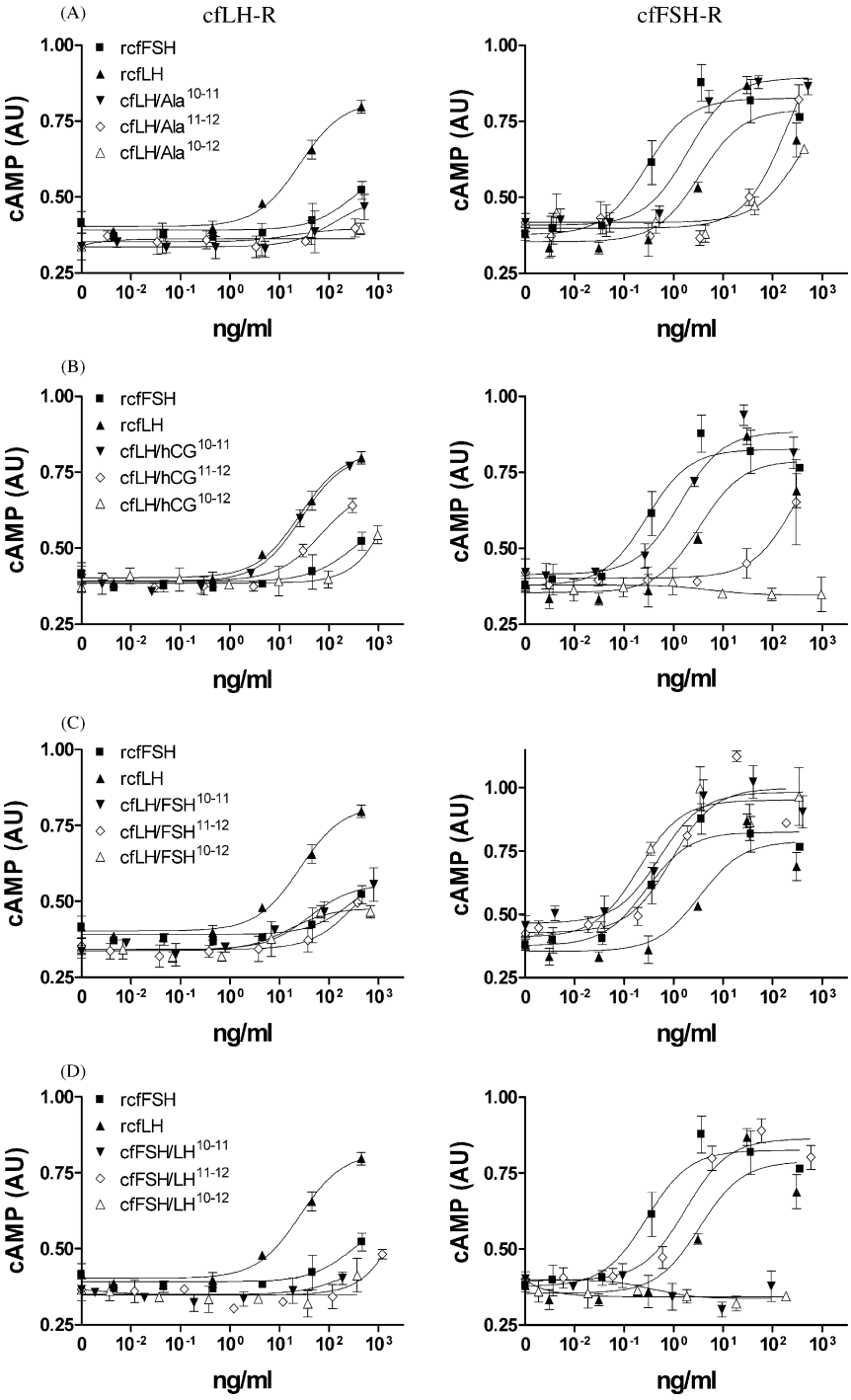

Fig. 2. Bioactivity of mutant cfLH and cfFSH β-subunit seat-belt analogs to induce cAMP production of cfLH-R and cfFSH-R, transiently expressed in HEK-T 293 cells. Effects of wild type recombinant cfFSH and cfLH, cfLH/Ala mutants (A), cfLH/hCG chimeras (B), cfLH/FSH chimeras (C) and cfFSH/LH chimeras (D) on the cAMP-mediated reporter gene activity in HEK-T 293 cells, transiently transfected with cfLH-R (left panel) or cfFSH-R (right panel). Results are shown as the mean ± S.E.M. of triplicate observations from a single representative experiment. Mean EC_{50} values are presented in Table 1.

tivated the cfLH-R in a similar way as wild type rcfFSH (Fig. 2D and Table 1). Only cfFSH/LH^{11-12} was able to significantly induce a receptor-mediated cAMP response in cfFSH-R expressing cells (Fig. 2D and Table 1), and had a similar potency to stimulate the cfFSH-R as rcfFSH.

4. Discussion

Glycoprotein hormones have coevolved together with their cognate receptors through gene duplications of ancestral β-subunit and receptor genes, respectively, followed by se-

quence divergence to define hormone-receptor pair selectivity (Moyle et al., 1994; Li and Ford, 1998; Moyle et al., 1998). However, functional duality of gonadotropins has not always evolved simultaneously with their structural divergence. To investigate if and how the seat-belt loop of cfLHβ allows the interaction of cfLH with both the cfLH-R and cfFSH-R, we systematically substituted the two successive cfLHβ intercysteine sequences in the seat-belt loop (i.e. sequences between the conserved Cys residues 10–11 and 11–12) with Ala-cassettes, or the corresponding cfFSHβ or hCGβ sequences. Replacing amino acid side chains with an Ala methyl-moiety would reveal if the seat-belt loop of cfLHβ is involved in directing cfFSH-R and/or cfLH-R recognition, whereas substitutions with corresponding cfFSHβ and hCGβ regions would be informative on how the β-subunit seat belt confers receptor specificity to cfLH. It should be noted that cfFSH as well as hCG are very specific in stimulating the cfFSH-R or cfLH-R, respectively (Vischer et al., 2003b; Vischer and Bogerd, 2003a). In addition, we introduced cfLHβ seat-belt loops into the cfFSHβ to investigate if they can confer cfLH-R and/or maintain cfFSH-R stimulating-activity to chimeric cfFSH analogs.

Concentrations of recombinant catfish gonadotropin analogs were determined by two different radioimmunoassays using polyclonal antisera against either the common cfGPα or intact cfLH. Typically, substitution of the $Cys^{11–12}$ region of cfLHβ with Ala and hCGβ residues resulted in cfLH analogs with lower intact hormone/cfGPα radioimmunoassay ratios as compared to wild type cfLH, suggesting that their overall conformation may have changed as a result of the mutations. This notion was supported by the fact that substitution of the cfLHβ $Cys^{11–12}$ region with corresponding FSHβ sequences did not severely alter the intact hormone/cfGPα ratio, indicating that the cfLH seat belt itself does not form a major epitope for anti-intact cfLH antisera binding. Typically, the overall conformation of human TSH/FSH/CG and equid CG analogs appeared not to be affected by interchange and/or mutagenesis of their respective seat-belt loops (e.g. Moyle et al., 1994; Dias et al., 1994; Lindau-Shepard et al., 1994; Grossmann et al., 1997; Chopineau et al., 2001). Crystal structures of hCG and hFSH revealed that the $Cys^{11–12}$ region of their β-subunit seat-belt loops are tightly wrapped around the third loop of the α-subunit (i.e. αL3; Lapthorn et al., 1994; Fox et al., 2001). Since the overall cfLH conformation appeared to be rather sensitive to substitutions of the $Cys^{11–12}$ region with Ala cassettes and corresponding hCG sequences, one may speculate that only the $Cys^{11–12}$ region of cfLHβ and cfFSHβ are tolerated in the vicinity of the αL3 of the cfLH analogs.

Ala-cassette substitution of the determinant loop of cfLHβ revealed that the determinant loop residues of cfLHβ (i.e. amino acids TMDTS, excluding D^{96}; see Fig. 1) is indispensable for cfLH-R-stimulating activity, but seems not to be involved in conferring cfFSH-R-stimulating activity to cfLH. The importance of the $Cys^{10–11}$ region of cfLHβ for its cfLH-R-stimulating activity was further supported by the substitution with its cfFSHβ counterpart. In addition, the introduction of the cfFSHβ determinant loop into cfLH resulted in an ∼18.5-fold increase in its cfFSH-R-stimulating activity compared to wild type cfLH, revealing the presence of important cfFSH-R-selective determinants in this region of cfFSHβ. In contrast, except for the conserved Asp residue (i.e. amino acid residue 93 of human FSHβ; see Fig. 3), the determinant loop of human FSHβ is not essential for FSH binding to the hFSH-R, but restrains the interaction with the hLH-R (Lindau-Shepard et al., 1994; Campbell et al., 1991; Moyle et al., 1994). The determinant loop of hCGβ, on the other hand, and in particular its net positive charge, was found to be crucial for LH-R-stimulating activity, without influencing FSH-R-recognition (Dias et al., 1994; Han et al., 1996). Surprisingly, the introduction of the cfLHβ determinant loop in cfFSHβ did not lead to an increased potency to stimulate the cfLH-R. Moreover the cfFSH/$LH^{10–11}$ analog was found to be devoid of both cfFSH-R- and cfLH-R-stimulating activity. In view of the fact that wild type cfLH displays intrinsic cfFSH-R-stimulating activity, it is unlikely that the cfLHβ determinant loop residues would be able to fully inhibit the potency to stimulate the cfFSH-R when introduced into cfFSH. Hence, we believe that the apparent absence of any bioactivity may be related to changes in overall conformation of cfFSH.

Replacing the cfLHβ determinant loop with the one of hCGβ did not change the cfLH-R-stimulating potency compared with wild type cfLH. In contrast to the situation in hCG (Han et al., 1996), this result suggests that the net charge of the determinant loop is of minor importance in directing cfLH-R-stimulating activity to cfLH. Since the determinant loops of cfLHβ and hCGβ share only a Thr residue, which corresponds to an Ile residue in cfFSHβ (Fig. 1), our observations predict a role for this hydrophylic side chain in mediating specific cfLH-R-stimulating activity. However, site-directed mutagenesis revealed that the corresponding Thr residue in hCG (i.e. Thr^{97}) is not involved in LH-R binding (Huang et al., 1993). Surprisingly, the hCGβ determinant loop also led to an increased cfFSH-R-stimulating potency of this cfLH analog. This contrasts earlier observations, in which the presence of the hCGβ determinant loop in the context of human FSHβ or TSHβ did not enhance their interaction with the FSH-R or TSH-R, respectively, but rather conferred bifunctionality to these chimeric analogs (Dias et al., 1994; Grossmann et al., 1997). Taking into account the receptor-binding profiles of these chimeric human glycoprotein hormone analogs, the large heterogeneity in physicochemical properties between the determinant loop side chains of hCGβ and cfFSHβ (see Fig. 1), and in particular the fact that hCG is devoid of cfFSH-R-stimulating activity (Bogerd et al., 2001), it is difficult to explain how the determinant loops of hCGβ and cfFSHβ exert a similar effect on cfFSH-R-stimulating activity when placed in the context of cfLH.

Thus, our findings suggest that the determinant loop of cfLHβ is essential for LH activity but seems to be dispensable for FSH activity. In this respect, the function of the de-

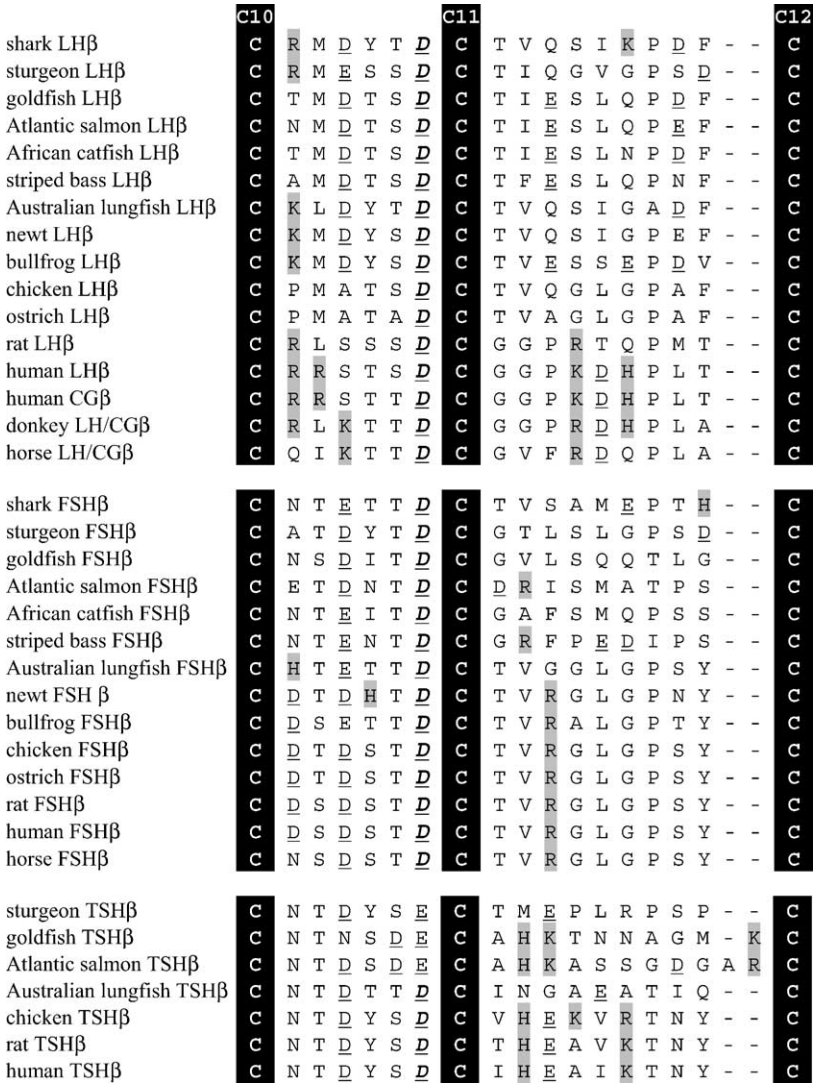

Fig. 3. Alignment of amino acid sequences of the seat-belt region of LH/CG, FSH and TSH β-subunits of species from various classes. Conserved cysteine residues are indicated by black boxes, whereas the conserved aspartic acid in the determinant loop is depicted in bold and italics. In addition, positively charged residues are indicated by grey boxes, while negatively charged residues are underlined.

terminant loop of fish LHβ appeared to be similar as that of mammalian LHβ/CGβ, directing the hormone's activity towards the LH-R. In human gonadotropins, the net charge difference between their determinant loops determines the ability of hCG and hFSH to interact with the LH-R (Campbell et al., 1991; Moyle et al., 1994; Han et al., 1996). Accurate comparison of superimposed crystal structures of hCG and hFSH revealed significant differences in surface charge characteristics between the hFSHβ and hCGβ determinant loops (Fox et al., 2001): three Asp residues in FSH form a negatively charged patch on one side of the determinant loop. On the other hand, the positively charged Arg residues in the determinant loop of hCGβ are not arranged as a charged patch, as their side chains are directed to opposite sides of the loop. The negatively charged surface of the determinant loop of hFSHβ is not essential for binding to the hFSH-R, but acts as an inhibitory determinant by blocking unintended interactions with hLH-R (Lindau-Shepard et al., 1994; Dias et al., 1994). Typically, the determinant loop of cfFSHβ appeared to be essential for cfFSH-R-stimulating activity, but seemed not to be actively involved in inhibiting the interaction with cfLH-R. Since determinant loops of fish LH and FSH share a similar negative net charge and positional conservation of acidic residues, of which the side chains are predicted to have a similar spatial orientation (cf. crystal structures of hCG and hFSH; Lapthorn et al., 1994; Fox et al., 2001), the mechanism by which key determinants in determinant loops of fish gonadotropins confer receptor-stimulating activity to these hormones remains puzzling.

The primary sequence of the Cys^{11-12} loop of cfLHβ seems to be important for both cfFSH-R- and cfLH-R-stimulating activity as indicated by Ala-cassette substitu-

tion. However, Ala-cassette mutation affected the potency to stimulate the cfLH-R more than its cfFSH-R stimulating activity. In contrast, replacing the Cys^{11-12} residues of cfLHβ with the corresponding hCGβ sequences affected the cfFSH-R-stimulating activity more than its potency to stimulate the cfLH-R. Similarly, introducing the Cys^{11-12} residues of hCGβ into human FSHβ significantly attenuated FSH-R-binding activity, however, without adding any LH-R-stimulating activity to this FSH analog (Dias et al., 1994). Nonetheless, it should be taken into account that substitution of the Cys^{11-12} residues of cfLHβ with Ala residues or the corresponding hCGβ residues may have changed the overall cfLH analog conformation, so that a direct effect of these mutations on receptor interaction can not be undoubtedly distinguished from an indirect effect resulting from altered hormone conformation. However, the fact that cfLH/Ala^{11-12} and cfLH/hCG^{11-12} had comparable cfFSH-R-stimulating activities, supports the importance of the primary sequence of the Cys^{11-12} region of cfLHβ in conferring cfFSH-R selectivity. The potency of cfLH to stimulate the cfFSH-R was significantly increased by replacing the Cys^{11-12} residues of cfLHβ with the corresponding cfFSHβ residues. Remarkably, however, the Cys^{11-12} region of cfLHβ was sufficient to confer full cfFSH-R-stimulating activity when placed in the context of cfFSH.

Although the Cys^{11-12} loop sequence has diverged considerably during the course of evolution between most LH/CG and FSH β-subunit paralogs, and in particular between orthologous β-subunits of fish and tetrapods (see Fig. 3), this region appeared to be of utmost importance for FSH activity in all species tested. Placing the human FSHβ Cys^{11-12} loop in the context of hCG, allowed this hCG analog to interact with the FSH-R (Campbell et al., 1991; Moyle et al., 1994), whereas substituting the FSHβ Cys^{11-12} loop with the corresponding hCGβ loop significantly impaired the binding to the FSH-R (Dias et al., 1994). Subsequent Ala-scanning analysis delineated the amino acid triplet Arg-Gly-Leu as being crucial for FSH-R binding (Lindau-Shepard et al., 1994). Likewise, the potency of horse LH/CG (eLH/CG; also known as pregnant mare serum gonadotropin) to promiscuously activate nonequine FSH-R, but not the horse FSH-R, relies exclusively upon the sequence of eCG/LHβ Cys^{11-12} loop (in particular Val-Phe-Arg; Chopineau et al., 2001). Typically, this equine-specific triplet can transfer additional FSH activity to donkey LH/CG, but was not sufficient enough to confer FSH-R-stimulating activity to hCG.

Based on the property to bind both FSH-R and LH-R, Moyle and co-workers (Campbell et al., 1991; Moyle et al., 1994; Han et al., 1996; Moyle et al., 1998) proposed that an hCGβ chimera that harbors its native, positively charged determinant loop in conjunction with the hFSHβ Cys^{11-12} region represents an omnipotent ancestral β-subunit. According to this model, LHβ/CGβ-like hormones and FSHβ/TSHβ-like hormones have evolved from this common ancestor, predominantly through sequence divergence of their seat-belt loops. In particular the introduction of negative charges into the determinant loop of FSHβ/TSHβ-like hormones would have attenuated their LH-R binding affinities. In contrast to this model, non-mammalian gonadotropins (i.e. LHβ and FSHβ) have net negatively charged determinant loops (Fig. 3). Together with the observed receptor promiscuity of chicken LH (Wakabayashi et al., 1997), bullfrog LH (Takada et al., 1986), and cfLH, phylogenetic data suggest that a presumed ancestral β-subunit had a negative charge and that neutrally or positively charged determinant loops were acquired during mammalian LHβ/CGβ evolution. Moreover, in contrast to the situation in mammals, our results indicate that charge differences in the determinant loop do not form the basis of the observed receptor selectivity in the catfish, which may rather reside in differences in hydrophobicity of the determinant loop.

Duality in gonadotropins is already established in elasmobranch fishes (Quérat et al., 2001), which have emerged before the divergence between actinopterygians (i.e. chondrostean and bony fish) and sarcopterygians (i.e. lungfishes and tetrapods) approximately 400 million years ago (Hedges and Kumar, 2003). Although phylogenetic analysis clusters all FSH, LH, and TSH β-subunit orthologs in separate branches (Vischer et al., 2003c; Quérat et al., 2004), striking differences are present between orthologous β-subunits when looking at their seat-belt loops (Fig. 3). Characteristic differences are the number of acidic residues in the determinant loop and the divergence in Cys^{11-12} loop sequence between actinopterygian and sarcopterygian FSH β-subunits. However, more significant are the net charge differences in the determinant loop and the differences in Cys^{11-12} loop sequence between non-mammalian and mammalian LH/(CG) β-subunits. Of particular interest is the high sequence identity in the Cys^{11-12} region between LHβ and FSHβ of amphibian and avian species, which may be associated with the observed FSH-R binding activity of chicken and bullfrog LH (Takada et al., 1986; Wakabayashi et al., 1997). Interestingly, the divergence between non-mammalian and mammalian LHβ/(CGβ) seat-belt loops seems to coincide with the necessity of a highly specific LH/CG, which came forth from a drastic change in female reproduction strategy. Whereas non-mammalian vertebrates are predominantly oviparous (i.e. females deposit unfertilized or developing eggs that complete their development and hatch in the external environment), mammals are generally viviparous characterized by an extended intrauterine pregnancy. The maintenance of an intrauterine pregnancy depends on a prolonged production of progesterone by the corpus luteum in response to LH/CG. In the ovary, primordial follicles grow spontaneously and randomly to become primary follicles that express FSH-R on their granulosa cells (Fauser and Van Heusden, 1997). In order to avoid undesired folliculogenesis during pregnancy as a result of the promiscuous activation of the FSH-R by elevated LH/CG levels, high receptor selectivity of mammalian LH/CG is an absolute requirement.

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